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IDENTIFICATION OF FREE-B-RING FLAVONOIDS AS POTENT COX-2 INHIBITORS

FIELD OF THE INVENTION

This invention relates generally to a method for the prevention and treatment of COX-2 mediated diseases and conditions. Specifically, the present invention relates to a method for the prevention and treatment of COX-2 mediated diseases and conditions by administration of compounds referred to herein as Free-B-Ring flavonoids. Included in this invention is an improved method to generate standardized Free-B-Ring flavonoid extracts from plant sources.

BACKGROUND OF THE INVENTION

The liberation and metabolism of arachidonic acid (AA) from the cell membrane, results in the generation of pro-inflammatory metabolites by several different pathways. Arguably, the two most important pathways to inflammation are mediated by the enzymes 5-lipoxygenase (5-LO) and cyclooxygenase (COX). These are parallel pathways that result in the generation of leukotrienes and prostaglandins, respectively, which play important roles in the initiation and progression of the inflammatory response. These vasoactive compounds are chemotaxins, which both promote infiltration of inflammatory cells into tissues and serve to prolong the inflammatory response. Consequently, the enzymes responsible for generating these mediators of inflammation have become the targets for many new anti-inflammatory drugs.

Inhibition of the enzyme cyclooxygenase (COX) is the mechanism of action attributed to most nonsteroidal anti-inflammatory drugs (NSAIDS). There are two distinct isoforms of the COX enzyme (COX-1 and COX-2) that share approximately 60% sequence homology, but differ in expression profiles and function. COX-1 is a constitutive form of the enzyme that has been linked to the production of physiologically important prostaglandins, which help regulate normal physiological functions, such as platelet aggregation, protection of cell function in the stomach and maintenance of normal kidney function. (Dannhardt and Kiefer (2001) Eur. J. Med. Chem. 36:109-26). The second isoform, COX-2, is a form of the enzyme

that is inducible by pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and other growth factors. (Herschmann (1994) Cancer Metastasis Rev. 134:241-56; Xie *et al.* (1992) Drugs Dev. Res. 25:249-65). This isoform catalyzes the production of prostaglandin E2 (PGE2) from arachidonic acid (AA). Inhibition of COX-2 is responsible for the anti-inflammatory activities of conventional NSAIDs.

Because the mechanism of action of COX-2 inhibitors overlaps with that of most conventional NSAID's, COX-2 inhibitors are used to treat many of the same symptoms, including pain and swelling associated with inflammation in transient conditions and chronic diseases in which inflammation plays a critical role. Transient conditions include treatment of inflammation associated with minor abrasions, sunburn or contact dermatitis, as well as, the relief of pain associated with tension and migraine headaches and menstrual cramps. Applications to chronic conditions include arthritic diseases, such as rheumatoid arthritis and osteoarthritis. Although, rheumatoid arthritis is largely an autoimmune disease and osteoarthritis is caused by the degradation of cartilage in joints, reducing the inflammation associated with each provides a significant increase in the quality of life for those suffering from these diseases. (Wienberg (2001) Immunol. Res. 22:319-41; Wollhiem (2000) Curr. Opin. Rheum. 13:193-201). In addition to rheumatoid arthritis, inflammation is a component of rheumatic diseases in general. Therefore, the use of COX inhibitors has been expanded to include diseases, such as systemic lupus erythromatosus (SLE) (Goebel *et al.* (1999) Chem. Res. Tox. 12:488-500; Patrono *et al.* (1985) J. Clin. Invest. 76:1011-1018), as well as, rheumatic skin conditions, such as scleroderma. COX inhibitors are also used for the relief of inflammatory skin conditions that are not of rheumatic origin, such as psoriasis, in which reducing the inflammation resulting from the over production of prostaglandins could provide a direct benefit. (Fogh *et al.* (1993) Acta Derm Venerologica 73:191-3). Simply stated COX inhibitors are useful for the treatment of symptoms of chronic inflammatory diseases, as well as, the occasional ache and pain resulting from transient inflammation.

In addition to their use as anti-inflammatory agents, another potential role for COX inhibitors is in the treatment of cancer. Over expression of COX-2 has been demonstrated in various human malignancies and inhibitors of COX-2 have been shown to be efficacious in

the treatment of animals with skin, breast and bladder tumors. While the mechanism of action is not completely defined, the over expression of COX-2 has been shown to inhibit apoptosis and increase the invasiveness of tumorgenic cell types. (Dempke *et al.* (2001) J. Can. Res. Clin. Oncol. 127:411-17; Moore and Simmons (2000) Current Med. Chem. 7:1131-44). It is possible that enhanced production of prostaglandins resulting from the over expression of COX-2 promotes cellular proliferation and consequently, increases angiogenesis. (Moore and Simmons (2000) Current Med. Chem. 7:1131-44; Fenton *et al.* (2001) Am. J. Clin. Oncol. 24:453-57).

There have been a number of clinical studies evaluating COX-2 inhibitors for potential use in the prevention and treatment of different type of cancers. Aspirin, a non-specific NSAID, for example, has been found to reduce the incidence of colorectal cancer by 40-50% (Giovannucci *et al.* (1995) N Engl J Med. 333:609-614) and mortality by 50% (Smalley *et al.* (1999) Arch Intern Med. 159:161-166). In 1999, the FDA approved the COX-2 inhibitor CeleCOXib for use in FAP (Familial Ademonatous Polyposis) to reduce colorectal cancer mortality. It is believed that other cancers, with evidence of COX-2 involvement, may be successfully prevented and/or treated with COX-2 inhibitors including, but not limited to esophageal cancer, head and neck cancer, breast cancer, bladder cancer, cervical cancer, prostate cancer, hepatocellular carcinoma and non-small cell lung cancer. (Jaeckel *et al.* (2001) Arch. Otolarnygol. 127:1253-59; Kirschenbaum *et al.* (2001) Urology 58:127-31; Dannhardt and Kiefer (2001) Eur. J. Med. Chem. 36:109-26). COX-2 inhibitors may also prove successful in preventing colon cancer in high-risk patients. There is also evidence that COX-2 inhibitors can prevent or even reverse several types of life-threatening cancers. To date, as many as fifty studies show that COX-2 inhibitors can prevent premalignant and malignant tumors in animals, and possibly prevent bladder, esophageal and skin cancers as well.

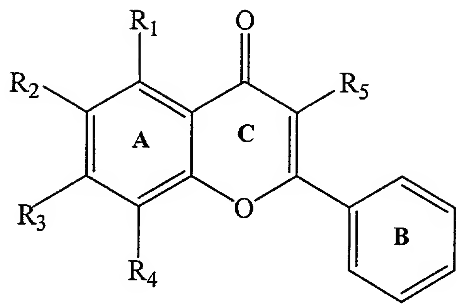
Recent scientific progress has identified correlations between COX-2 expression, general inflammation and the pathogenesis of Alzheimer's Disease (AD). (Ho *et al.* (2001) Arch. Neurol. 58:487-92). In animal models, transgenic mice that over express the COX-2 enzyme have neurons that are more susceptible to damage. The National Institute on Aging

(NIA) is launching a clinical trial to determine whether NSAIDs can slow the progression of Alzheimer's Disease. Naproxen (a non-selective NSAID) and rofeCOXib (Vioxx, a COX-2 specific selective NSAID) will be evaluated. Previous evidence has indicated inflammation contributes to Alzheimer's Disease. According to the Alzheimer's Association and the NIA, about 4 million people suffer from AD in the U.S.; and this is expected to increase to 14 million by mid-century.

The COX enzyme (also known as prostaglandin H2 synthase) catalyzes two separate reactions. In the first reaction, arachidonic acid is metabolized to form the unstable prostaglandin G2 (PGG2), a cyclooxygenase reaction. In the second reaction, PGG2 is converted to the endoperoxide PGH2, a peroxidase reaction. The short-lived PGH2 non-enzymatically degrades to PGE2. The compounds described herein are the result of a discovery strategy that combined an assay focused on the inhibition of COX-1 and COX-2 peroxidase activity with a chemical dereplication process to identify novel inhibitors of the COX enzymes.

Flavonoids are a widely distributed group of natural products. The intake of flavonoids has been demonstrated to be inversely related to the risk of incident dementia. The mechanism of action, while not known, has been speculated as being due to the anti-oxidative effects of flavonoids. (Commenges *et al.* (2000) Eur. J. Epidemiol 16:357-363). Polyphenol flavones induce programmed cell death, differentiation, and growth inhibition in transformed colonocytes by acting at the mRNA level on genes including COX-2, Nf kappaB and bcl-X(L). (Wenzel *et al.* (2000) Cancer Res. 60:3823-3831). It has been reported that the number of hydroxyl groups on the B ring is important in the suppression of COX-2 transcriptional activity. (Mutoh *et al.* (2000) Jnp. J. Cancer Res. 91:686-691).

Free-B-Ring flavones and flavonols are a specific class of flavonoids, which have no substituent groups on the aromatic B ring, as illustrated by the following general structure:



wherein

R_1, R_2, R_3, R_4, and R_5 are independently selected from the group consisting of -H, -OH, -SH, OR, -SR, $-NH_2$, -NHR, $-NR_2$, $-NR_3^+X^-$, a carbon, oxygen, nitrogen or sulfur, glycoside of a single or a combination of multiple sugars including, but not limited to aldopentoses, methyl-aldopentose, aldohexoses, ketohexose and their chemical derivatives thereof;

wherein

R is an alkyl group having between 1-10 carbon atoms; and

X is selected from the group of pharmaceutically acceptable counter anions including, but not limited to hydroxyl, chloride, iodide, sulfate, phosphate, acetate, fluoride, carbonate, etc.

Free-B-Ring flavonoids are relatively rare. Out of a total 9396 flavonoids synthesized or isolated from natural sources, only 231 Free-B-Ring flavonoids are known. (The Combined Chemical Dictionary, Chapman & Hall/CRC, Version 5:1 June 2001).

The Chinese medicinal plant, *Scutellaria baicalensis* contains significant amounts of Free-B-Ring flavonoids, including baicalein, baicalin, wogonin and baicalenoside. Traditionally, this plant has been used to treat a number of conditions including clearing away heat, purging fire, dampness-warm and summer fever syndromes; polydipsia resulting from high fever; carbuncle, sores and other pyogenic skin infections; upper respiratory infections, such as acute tonsillitis, laryngopharyngitis and scarlet fever; viral hepatitis; nephritis; pelvitis; dysentery; hematemesis and epistaxis. This plant has also traditionally been used to prevent miscarriage. (See Encyclopedia of Chinese Traditional Medicine, ShangHai Science and Technology Press, ShangHai, China, 1998). Clinically *Scutellaria* is now used to treat

conditions such as pediatric pneumonia, pediatric bacterial diarrhea, viral hepatitis, acute gallbladder inflammation, hypertension, topical acute inflammation, resulting from cuts and surgery, bronchial asthma and upper respiratory infections (Encyclopedian of Chinese Traditional Medicine, ShangHai Science and Technology Press, ShangHai, China, 1998). The pharmacological efficacy of Scutellaria roots for treating bronchial asthma is reportedly related to the presence of Free-B-Ring flavonoids and their suppression of eotaxin associated recruitment of eosinophils. (Nakajima *et al.* (2001) Planta Med. 67(2):132-135).

Free-B-Ring flavonoids have been reported to have diverse biological activity. For example, galangin (3,5,7-trihydroxyflavone) acts as anti-oxidant and free radical scavenger and is believed to be a promising candidate for anti-genotoxicity and cancer chemoprevention. (Heo *et al.* (2001) Mutat. Res. 488(2):135-150). It is an inhibitor of tyrosinase monophenolase (Kubo *et al.* (2000) Bioorg. Med. Chem. 8(7):1749-1755), an inhibitor of rabbit heart carbonyl reductase (Imamura *et al.* (2000) J. Biochem. 127(4):653-658), has antimicrobial activity (Afolayan and Meyer (1997) Ethnopharmacol. 57(3):177-181) and antiviral activity (Meyer *et al.* (1997) J. Ethnopharmacol. 56(2):165-169). Baicalein and galangin, two other Free-B-Ring flavonoids, have antiproliferative activity against human breast cancer cells. (So *et al.* (1997) Cancer Lett. 112(2):127-133).

Typically, flavonoids have been tested for activity randomly based upon their availability. Occasionally, the requirement of substitution on the B-ring has been emphasized for specific biological activity, such as the B-ring substitution required for high affinity binding to p-glycoprotein (Boumendjel *et al.* (2001) Bioorg. Med. Chem. Lett. 11(1):75-77); cardiotonic effect (Itoigawa *et al.* (1999) J. Ethnopharmacol. 65(3): 267-272), protective effect on endothelial cells against linoleic acid hydroperoxide-induced toxicity (Kaneko and Baba (1999) Biosci Biotechnol. Biochem 63(2):323-328), COX-1 inhibitory activity (Wang (2000) Phytomedicine 7:15-19) and prostaglandin endoperoxide synthase (Kalkbrenner *et al.* (1992) Pharmacology 44(1):1-12). Only a few publications have mentioned the significance of the unsubstituted B ring of the Free-B-Ring flavonoids. One example, is the use of 2-phenyl flavones, which inhibit NAD(P)H quinone acceptor oxidoreductase, as potential anticoagulants. (Chen *et al.* (2001) Biochem. Pharmacol. 61(11):1417-1427).

The reported mechanism of action related to the anti-inflammatory activity of various Free-B-Ring flavonoids has been controversial. The anti-inflammatory activity of the Free-B-Ring flavonoids, chrysin (Liang *et al.* (2001) FEBS Lett. 496(1):12-18), wogonin (Chi *et al.* (2001) Biochem. Pharmacol. 61:1195-1203) and halangin (Raso *et al.* (2001) Life Sci. 68(8):921-931), has been associated with the suppression of inducible cyclooxygenase and nitric oxide synthase via activation of peroxisome-proliferator activated receptor gamma and influence on degranulation and AA release. (Tordera *et al.* (1994) Z. Naturforsch [C] 49:235-240). It has been reported that oroxylin, baicalein and wogonin inhibit 12-lipoxygenase activity without affecting cyclooxygenase. (You *et al.* (1999) Arch. Pharm. Res. 22(1):18-24). More recently, the anti-inflammatory activity of wogonin, baicalin and baicalein has been reported as occurring through inhibition of inducible nitric oxide synthase and COX-2 gene expression induced by nitric oxide inhibitors and lipopolysaccharide. (Chen *et al.* (2001) Biochem. Pharmacol. 61(11):1417-1427). It has also been reported that oroxylin acts via suppression of nuclear factor-kappa B activation. (Chen *et al.* (2001) Biochem. Pharmacol. 61(11):1417-1427). Finally, wogonin reportedly inhibits inducible PGE2 production in macrophages. (Wakabayashi and Yasui (2000) Eur. J. Pharmacol. 406(3):477-481). Inhibition of the phosphorylation of mitrogen-activated protein kinase and inhibition of Ca^{2+} ionophore A23187 induced prostaglandin E2 release by baicalein has been reported as the mechanism of anti-inflammatory activity of *Scutellariae Radix.* (Nakahata *et al.* (1999) Nippon Yakurigaku Zasshi, 114, Supp. 11:215P-219P; Nakahata *et al.* (1998) Am. J. Chin Med. 26:311-323). Baicalin from *Sculettaria baicalensis,* reportedly inhibits superantigenic staphylococcal exotoxins stimulated T-cell proliferation and production of IL-1β, interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). (Krakauer *et al.* (2001) FEBS Lett. 500:52-55). Thus, the anti-inflammatory activity of baicalin has been associated with inhibiting the proinflammatory cytokines mediated signaling pathways activated by superantigens. However, it has also been proposed that the anti-inflammatory activity of baicalin is due to the binding of a variety of chemokines, which limits their biological activity. (Li *et al.* (2000) Immunopharmacology 49:295-306). Recently, the effects of baicalin on adhesion molecule expression induced by thrombin and thrombin receptor agonist peptide

(Kimura *et al.* (2001) Planta Med. 67:331-334), as well as, the inhibition of mitogen-activated protein kinase cascade (MAPK) (Nakahata *et al.* (1999) Nippon Yakurigaku Zasshi, 114, Supp 11:215P-219P; Nakahata *et al.* (1998) Am. J. Chin Med. 26:311-323) have been reported. To date there have been no reports that link Free-B-Ring flavonoids with COX-2 inhibitory activity.

To date, a number of naturally occurring Free-B-Ring flavonoids have been commercialized for varying uses. For example, liposome formulations of Scutellaria extracts have been utilized for skin care (U.S. Patent Nos. 5,643,598; 5,443,983). Baicalin has been used for preventing cancer, due to its inhibitory effects on oncogenes (U.S. Patent No. 6,290,995). Baicalin and other compounds have been used as antiviral, antibacterial and immunomodulating agents (U.S. Patent No. 6,083,921) and as natural anti-oxidants (Poland Pub. No. 9,849,256). Chrysin has been used for its anxiety reducing properties (U.S. Patent No. 5,756,538). Anti-inflammatory flavonoids are used for the control and treatment of anorectal and colonic diseases (U.S. Patent No. 5,858,371), and inhibition of lipoxygenase (U.S. Patent No. 6,217,875). Flavonoid esters constitute active ingredients for cosmetic compositions (U.S. Patent No. 6,235,294).

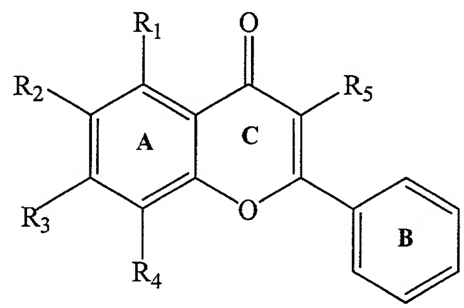
Japanese Patent No. 63027435, describes the extraction, and enrichment of baicalein and Japanese Patent No. 61050921 describes the purification of baicalin.

SUMMARY OF THE INVENTION

The present invention includes methods that are effective in inhibiting the cyclooxygenase enzyme COX-2. The method for inhibiting the cyclooxygenase enzyme COX-2 is comprised of administering a composition comprising a Free-B-Ring flavonoid or a composition containing a mixture of Free-B-Ring flavonoids to a host in need thereof.

The present also includes methods for the prevention and treatment of COX-2 mediated diseases and conditions. The method for preventing and treating COX-2 mediated diseases and conditions is comprised of administering to a host in need thereof an effective amount of a composition comprising a Free-B-Ring flavonoid or a composition containing a mixture of Free-B-Ring flavonoids and a pharmaceutically acceptable carrier.

The Free-B-Ring flavonoids that can be used in accordance with the following include compounds illustrated by the following general structure:



wherein

R_1, R_2, R_3, R_4, and R_5 are independently selected from the group consisting of -H, -OH, -SH, OR, -SR, $-NH_2$, -NHR, $-NR_2$, $-NR_3^+X^-$, a carbon, oxygen, nitrogen or sulfur, glycoside of a single or a combination of multiple sugars including, but not limited to aldopentoses, methyl-aldopentose, aldohexoses, ketohexose and their chemical derivatives thereof;

wherein

R is an alkyl group having between 1-10 carbon atoms; and

X is selected from the group of pharmaceutically acceptable counter anions including, but not limited to hydroxyl, chloride, iodide, sulfate, phosphate, acetate, fluoride, carbonate, etc.

The method of this invention can be used to treat and prevent a number of COX-2 mediated diseases and conditions including, but not limited to, osteoarthritis, rheumatoid arthritis, menstrual cramps, systemic lupus erythromatosus, psoriasis, chronic tension headaches, migraine headaches, topical wound and minor inflammatory conditions, inflammatory bowel disease and solid cancers.

The Free-B-Ring flavonoids of this invention may be obtained by synthetic methods or extracted from the family of plants including, but not limited to Annonaceae, Asteraceae, Bignoniaceae, Combretaceae, Compositae, Euphorbiaceae, Labiatae, Lauranceae, Leguminosae, Moraceae, Pinaceae, Pteridaceae, Sinopteridaceae, Ulmaceae and Zingiberacea.

The Free-B-Ring flavonoids can be extracted, concentrated, and purified from the following genus of high plants, including but not limited to Desmos, Achyrocline, Oroxylum, Buchenavia, Anaphalis, Cotula, Gnaphalium, Helichrysum, Centaurea, Eupatorium, Baccharis, Sapium, Scutellaria, Molsa, Colebrookea, Stachys, Origanum, Ziziphora, Lindera, Actinodaphne, Acacia, Derris, Glycyrrhiza, Millettia, Pongamia, Tephrosia, Artocarpus, Ficus, Pityrogramma, Notholaena, Pinus, Ulmus and Alpinia.

The compositions of this invention can be administered by any method known to one of ordinary skill in the art. The modes of administration include, but are not limited to, enteral (oral) administration, parenteral (intravenous, subcutaneous, and intramuscular) administration and topical application. The method of treatment according to this invention comprises administering internally or topically to a patient in need thereof a therapeutically effective amount of the individual and/or a mixture of multiple Free-B-Ring flavonoids from a single source or multiple sources that include, but not limited to, synthetically obtained, naturally occurring, or any combination thereof.

This invention includes an improved method for isolating and purifying Free-B-Ring flavonoids from plants containing these compounds. The method of the present invention comprises: a) extracting the ground biomass of a plant containing Free-B-Ring flavonoids; b) neutralizing and concentrating said extract; and c) purifying said neutralized and concentrated extract. In a preferred embodiment of the invention the extract is purified using a method selected from the group consisting of recrystallization, precipitation, solvent partition and/or chromatographic separation. The present invention provides a commercially viable process for the isolation and purification of Free-B-Ring flavonoids having desirable physiological activity.

The present invention implements a strategy that combines a series of biomolecular screens with a chemical dereplication process to identify active plant extracts and the particular compounds within those extracts that specifically inhibit COX-2 enzymatic activity and inflammation. A total of 1230 plant extracts were screened for their ability to inhibit the peroxidase activity associated with recombinant COX-2. This primary screen identified 22 plant extracts that were further studied for their ability to specifically and selectively inhibit

COX-2 *in vitro* in both cell based and whole blood assays. Those extracts that were efficacious *in vitro* were then tested for their ability to inhibit inflammation *in vivo* using a both air pouch and topical ear-swelling models of inflammation when administered by multiple routes (IP and oral). These studies resulted in the discovery of botanical extracts that inhibited COX-2 activity and were efficacious both *in vitro* and *in vivo.* These studies also resulted in the identification of specific Free-B-Ring flavonoids associated with COX-2 inhibition in each of these extracts. Applicant believes that this is first report of a correlation between Free-B-Ring flavonoid structure and COX-2 inhibitory activity.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts graphically the inhibition of COX-1 and COX-2 by HTP fractions from *Scutellaria baicaensis*. The extracts were prepared and fractionated as described in Examples 1 and 3. The extracts were examined for their inhibition of the peroxidase activity of recombinant ovine COX-1 (■) or ovine COX-2 (♦). The data is presented as percent of untreated control.

Figure 2 depicts the high pressure liquid chromatography (HPLC) chromatograms of Free-B-Ring Flavonoids in organic extracts from *Scutellaria lateriflora* roots (Figure 2A), *Scutellaria orthocalyx* roots (Figure 2B) and *Scutellaria baicaensis* roots (Figure 2C).

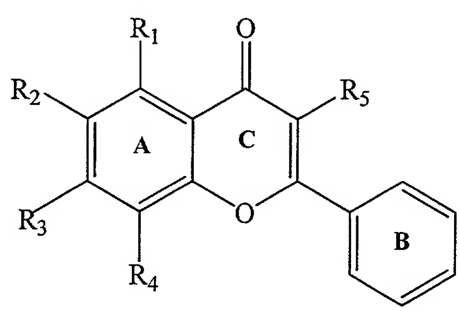
Figure 3 demonstrates the *in vivo* efficacy of Free-B-Ring Flavonoids from *Scutellaria baicaensis* on arachidonic acid induced inflammation. The *in vivo* efficacy was evaluated based on the ability to inhibit swelling induced by direct application of arachidonic acid as described in Example 9. The average differences in swelling between the treated ears and control ears are represented in Figure 3A. Figure 3B demonstrates the percent inhibition of each group in comparison to the arachidonic acid treated control.

Figure 4 illustrates the *in vivo* efficacy of Free-B-Ring Flavonoids isolated from *Scutellaria baicalensis* on inflammation induced by Zymosan. Zymosan was used to elicit a pro-inflammatory response in an air pouch as described in Example 9. Markers of inflammation including infiltration of pro-inflammatory cells (Figure 4A), percent inhibition of MPO activity with in the air pouch fluid (Figure 4B), and percent inhibition of TNF-α production (Figure 4C) were used to evaluate the efficacy and mechanism of action of the anti-inflammatory activity of the Free-B-Ring Flavonoids from *Scutellaria baicalensis*.

DETAILED DESCRIPTION OF THE INVENTION

Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided.

"Free-B-Ring Flavonoids" as used herein are a specific class of flavonoids, which have no substituent groups on the aromatic B ring, as illustrated by the following general structure:



wherein

R_1, R_2, R_3, R_4, and R_5 are independently selected from the group consisting of -H, -OH, -SH, OR, -SR, $-NH_2$, -NHR, $-NR_2$, $-NR_3^+X^-$, a carbon, oxygen, nitrogen or sulfur, glycoside of a single or a combination of multiple sugars including, but not limited to aldopentoses, methyl-aldopentose, aldohexoses, ketohexose and their chemical derivatives thereof;

wherein

R is an alkyl group having between 1-10 carbon atoms; and

X is selected from the group of pharmaceutically acceptable counter anions including, but not limited to hydroxyl, chloride, iodide, sulfate, phosphate, acetate, fluoride, carbonate, etc.

"Therapeutic" as used herein, includes treatment and/or prophylaxis. When used, therapeutic refers to humans, as well as, other animals.

"Pharmaceutically or therapeutically effective dose or amount" refers to a dosage level sufficient to induce a desired biological result. That result may be the alleviation of the signs, symptoms or causes of a disease or any other desirous alteration of a biological system.

A **"host"** is a living subject, human or animal, into which the compositions described herein are administered.

Note, that throughout this application various citations are provided. Each citation is specifically incorporated herein in its entirety by reference.

The present invention includes methods that are effective in inhibiting the cyclooxygenase enzyme COX-2. The method for inhibiting the cyclooxygenase enzyme COX-2 is comprised of administering a composition comprising a Free-B-Ring flavonoid or a composition containing a mixture of Free-B-Ring flavonoids to a host in need thereof.

The present also includes methods for the prevention and treatment of COX-2 mediated diseases and conditions. The method for preventing and treating COX-2 mediated diseases and conditions is comprised of administering to a host in need thereof an effective amount of a composition comprising a Free-B-Ring flavonoid or a composition containing a mixture of Free-B-Ring flavonoids and a pharmaceutically acceptable carrier.

The Free-B-Ring flavonoids that can be used in accordance with the following include compounds illustrated by the general structure set forth above. The Free-B-Ring flavonoids of this invention may be obtained by synthetic methods or may be isolated from the family of plants including, but not limited to Annonaceae, Asteraceae, Bignoniaceae, Combretaceae, Compositae, Euphorbiaceae, Labiatae, Lauranceae, Leguminosae, Moraceae, Pinaceae, Pteridaceae, Sinopteridaceae, Ulmaceae, and Zingiberacea. The Free-B-Ring flavonoids can be extracted, concentrated, and purified from the following genus of high plants, including but

not limited to Desmos, Achyrocline, Oroxylum, Buchenavia, Anaphalis, Cotula, Gnaphalium, Helichrysum, Centaurea, Eupatorium, Baccharis, Sapium, Scutellaria, Molsa, Colebrookea, Stachys, Origanum, Ziziphora, Lindera, Actinodaphne, Acacia, Derris, Glycyrrhiza, Millettia, Pongamia, Tephrosia, Artocarpus, Ficus, Pityrogramma, Notholaena, Pinus, Ulmus, and Alpinia.

The flavonoids can be found in different parts of plants, including but not limited to stems, stem barks, twigs, tubers, roots, root barks, young shoots, seeds, rhizomes, flowers and other reproductive organs, leaves and other aerial parts.

In order to identify compounds able to inhibit the COX enzymes an extract library composed of 1230 extracts from 615 medicinal plants collected from China, India, and other countries was created. A general method for preparing the extracts is described in Example 1, which uses the Scutellaria species for purposes of illustration. The extraction process yields an organic and an aqueous extract for each species examined. The results of the extraction of various Scutellaria species are set forth in Table 1. These primary extracts are the source material used in the preliminary assay to identify inhibitors of the cyclooxygenase enzyme's peroxidase activity, which is one of the main functional activities of cyclooxygenase and is responsible for converting PGG2 to PGH2 and ultimately PGE2, as described in detail above. This assay is described in Example 2 and the results are set forth in Table 2. With reference to Table 2, it can be seen that two species of Scutellaria and three other plant species, all of which contain Free-B-ring flavonoids as common components, showed inhibitory activity in the primary screen against the peroxidase activity of COX-2 albeit to differing degrees. The COX-2 inhibitory activity is found predominantly in the organic extracts, which contain the most of medium polarity Free-B-Ring flavonoids.

The COX-2 inhibitory activity from the primary assay of the crude extracts has been confirmed by measurement of dose response and IC_{50} (the concentration required to inhibit 50% of the enzyme's activity). The IC_{50} values are set forth in Table 3. As can be seen in Table 3, in this assay *Scutellaria orthocalyx* root extract and *Murica nana* leaf extract were the most efficacious (IC_{50}= 6-10 mg/mL). Extracts from *Scutellaria sp.* that demonstrated the greatest selectivity against COX-2 were *Scutellaria lateriflora* (COX-2 IC_{50}: 30 mg/mL;

COX-1 IC_{50}: 80 mg/mL). Thus, the primary screen for inhibitors of the COX enzyme identified five extracts containing Free-B-Ring flavonoids that were efficacious *in vitro* and some of which demonstrated specificity for the COX-2 enzyme.

In order to efficiently identify active compounds from plant extracts, a high throughput fractionation process was used, as described in Example 3. Briefly, the active organic and aqueous extracts were fractionated using two different methodologies, respectively. The fractions were collected in a 96 deep well plate. Each of the fractions was tested for its ability to inhibit COX activity as per the primary assay, as described in Example 4. The results are set forth in Figure 1, which depicts the profile of COX-1 and COX-2 inhibition by various HTP fractions derived from the roots *of Scutellaria baicalensis*. As can be seen in Figure 1, the most potent COX inhibitory activity was found in two major fractions, E11 and F11. It should be noted that three separate HTP fractions actually exhibit inhibitory activity, suggesting that there are multiple compounds contributing to the observed inhibitory effects of the whole extract.

The separation, purification and identification of the active Free-B-Ring flavonoids present in the organic extract of *Scutellaria orthocalyx* is described in Example 5. Using the methodology described in Example 5, baicalein was identified as the major active component in the organic extract from the roots of *Scutellaria orthocalyx*. As shown in the Example 6, several other Free-B-Ring flavonoids have been isolated and tested for inhibition of COX-1 and COX-2 enzymatic activity. The results are set forth in Table 4.

Example 7 and Table 5 set forth the content and quantity of the Free-B-Ring flavonoids in five active plant extracts from three different species of plants. The Free-B-Ring flavonoids are present in much greater amounts in the organic extracts verses the aqueous extracts. This explains why the COX-2 inhibitory activity has usually shown up in the organic extracts rather than the aqueous extracts.

The primary assay described in Example 2 to identify active extracts is a cell free system utilizing recombinant enzymes. To further demonstrate the biological activity of the active extracts and compounds, two models that represent cell based *in vitro* efficacy and animal based *in vivo* efficacy were employed. The method used to evaluate *in vitro* efficacy

and selectivity is described in Example 8. Two cell lines were selected that could be induced to express primarily COX-1 (THP-1 cells) and COX-2 (HOSC cells), respectively. Each cell type was examined for the production of PGE2, the primary product of the COX enzymes. The results are set forth in Table 6, which shows that three organic extracts from three different species of *Scutellaria* showed inhibition of both the COX-1 and COX-2 enzymes with a preference for the COX-1 enzyme. While the use of the THP-1 cell line is important and demonstrates the ability of the active compounds to cross the cell membrane, it is an immortalized cell line, therefore evaluation of the efficacy of Free-B-Ring flavonoids based on a more relevant model system is desirable. As a result, the extracts were also evaluated using whole blood as the primary source of both COX-1 and COX-2 activity. This system measures the inhibition of the production of PGE2 vs. TXB_2 to differentiate between COX-2 and COX-1 inhibitory activity, respectively. The results, which are set forth in Table 6 demonstrate that both the COX-1 and COX-2 enzymes are inhibited by the Free-B-Ring flavonoids from all three *Scutellaria* root extracts. The IC_{50} values suggest that within this system all Free-B-Ring flavonoids, except those from *Scutellaria baicaensis* are more efficacious against COX-2. Taken as a whole, the inhibitory effect of the active compounds within these extracts is significant and efficacious in both cell free and cell-based systems *in vitro*. Also, no cell toxicity been observed in the testing process.

Two separate *in vivo* models were employed to determine whether the *in vitro* efficacy observed from the Free-B-Ring flavonoids translated to an ability to inhibit *in vivo* inflammatory responses. The two models are described in Example 9. The first of these systems was designed to measure inflammation resulting directly from the arachidonic acid metabolism pathway. In this example, mice were treated with Free-B-Ring flavonoids from three *Scutellaria* species prior to the topical application of AA to the ear to induce the inflammatory response. The effect of pretreating the animals was then measured by the inhibition of the ear swelling using a micrometer. The Free-B-Ring flavonoids containing extracts from these three *Scutellaria* species demonstrated varying degrees of efficacy. For example, the Free-B-Ring flavonoids extracted from the roots of *Scutellaria baicaensis* inhibited ear swelling by 60% in comparison to controls when delivered by both oral and IP

routes as illustrated in Figures 3A and B. This is the similar to the degree of inhibition seen with the positive control indomethacin, when delivered IP at a concentration of 5 mg/kg. Free-B-Ring flavonoids extracted from *Scutellaria orthocalyx* were efficacious when delivered by IP routes of administration, but had no effect when delivered by oral routes and finally the Free-B-Ring flavonoids extracted from *Scutellaria lateriflora* showed no effect regardless of the route of administration (data not shown).

The Free-B-Ring flavonoids isolated from *Scutellaria baicaensis* have been clearly demonstrated to be the most efficacious against inflammation induced directly by the arachidonic acid. Therefore, the efficacy of these Free-B-Ring flavonoids was examined using a second model in which multiple mechanisms of inflammation are ultimately responsible for the final effect. This system is therefore more relevant to naturally occurring inflammatory responses. Using this model, a very potent activator of the complement system is injected into an air pouch created on the back of Balb/C mice. This results in a cascade of inflammatory events including, infiltration of inflammatory cells, activation of COX enzymes, resulting in the release of PGE_2, the enzyme myeloperoxidase (MPO), and production of a very specific profile of pro-inflammatory cytokines including TNF-α. These studies demonstrated that even though the Free-B-Ring flavonoids isolated from *Scutellaria baicaensis* did not inhibit the initial infiltration (chemotactic response) of inflammatory cells into the air pouch, they blocked the activation of those cells. This is evidenced by the lack of MPO excreted into the extracellular fluid of the pouch and the noted lack of production of TNF-α. The results are set forth in Figure 4. The data demonstrates that the Free-B-Ring flavonoids are efficacious and help control an inflammatory response in a model system where multiple inflammatory pathways are active.

The preparation of products for administration in pharmaceutical preparations may be performed by a variety of methods well known to those skilled in the art. The Free-B-Ring flavonoids may be formulated as an herb powder in the form of its natural existence; as solvent and/or supercritical fluid extracts in different concentrations; as enriched and purified compounds through recrystallization, column separation, solvent partition, precipitation and

other means, as a pure and/or a mixture containing substantially purified Free-B-Ring flavonoids prepared by synthetic methods.

Various delivery systems are known in the art and can be used to administer the therapeutic compositions of the invention, e.g., aqueous solution, encapsulation in liposomes, microparticles, and microcapsules.

Therapeutic compositions of the invention may be administered parenterally by injection, although other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one preferred embodiment, it is envisioned that the carrier and Free-B-Ring flavonoid(s) constitute a physiologically-compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release or absorption of the ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parental administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder; or directly capsulated and/or tableted with other inert carriers for oral administration. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The manner of administering formulations containing the compositions for systemic delivery may be via enteral, subcutaneous, intramuscular, intravenous, intranasal or vaginal or rectal suppository.

The amount of the composition that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder of condition, which can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may

optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness or advancement of the disease or condition, and should be decided according to the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curved derived from *in vitro* or animal model test systems. For example, an effective amount of the composition of the invention is readily determined by administering graded doses of the composition of the invention and observing the desired effect.

The method of treatment according to this invention comprises administering internally or topically to a patient in need thereof a therapeutically effective amount of the individual and/or a mixture of multiple Free-B-Ring flavonoids from a single source or multiple sources. The purity of the individual and/or a mixture of multiple Free-B-Ring flavonoids includes, but is not limited to 0.01% to 100%, depending on the methodology used to obtain the compound(s). In a preferred embodiment doses of the Free-B-Ring flavonoids and pharmaceutical compositions containing that same are an efficacious, nontoxic quantity generally selected from the range of 0.01 to 200 mg/kg of body weight. Persons skilled in the art using routine clinical testing are able to determine optimum doses for the particular ailment being treated.

This invention includes an improved method for isolating and purifying Free-B-Ring flavonoids from plants, which is described in Example 10. In Example 10, Free-B-Ring flavonoids from two Scutellaria species were extracted with different solvent systems. The results are set forth in Tables 7 and 8. The improved method of this invention comprises: extraction of the ground biomass of a plant containing Free-B-Ring flavonoids with single or combination of organic solvent and/or water; neutralization and concentration of the neutralized extract; and purification of said extract by recrystallization and/or chromatography. As provided above, these Free-B-Ring flavonoids can be isolated from the genera of more than twenty plant families. The method of this invention can be extended to the isolation of these compounds from any plant source containing these compounds.

Additionally the Free-B-Ring flavonoids can be isolated from various parts of the plant including, but not limited to, the whole plant, stems, stem bark, twigs, tubers, flowers, fruit,

roots, root barks, young shoots, seeds, rhizomes and aerial parts. In a preferred embodiment the Free-B-Ring flavonoids are isolated from the roots, reproductive organs or the whole plant.

The solvent used for extraction of the ground biomass of the plant includes, but is not limited to water, acidified water, water in combination with miscible hydroxylated organic solvent(s) including, but not limited to, methanol or ethanol and an mixture of alcohols with other organic solvent(s) such as THF, acetone, ethyl acetate etc. In one embodiment the extract is neutralized to a pH of 4.5-5.5 and then concentrated and dried to yield a powder. The Free-B-Ring flavonoids can then be purified by various methods including, but not limited to recrystallization, solvent partition, precipitation, sublimation, and/or chromatographic methods including, but not limited to, ion exchange chromatography, absorption chromatography, reverse phase chromatography, size exclusive chromatography, ultra-filtration or a combination of thereof.

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLES

Example 1. Preparation of Organic and Aqueous Extracts from Scutellaria plants

Plant material from *Scutellaria orthocalyx* roots, *Scutellaria baicaensis* roots or *Scutellaria lateriflora* whole plant was ground to a particle size of no larger than 2 mm. Dried ground plant material (60 g) was then transferred to an Erlenmeyer flask and methanol:dichloromethane (1:1) (600 mL) was added. The mixture was shaken for one hour, filtered and the biomass was extracted again with methanol:dichloromethane (1:1) (600 mL). The organic extracts were combined and evaporated under vacuum to provide the organic extract (see Table 1 below). After organic extraction, the biomass was air dried and extracted once with ultra pure water (600 mL). The aqueous solution was filtered and freeze-dried to provide the aqueous extract (see Table 1 below).

Table 1. Yield of Organic and Aqueous Extracts of various *Scutellaria* species

Plant Source	Amount	Organic Extract	Aqueous Extract
Scutellaria orthocalyx roots	60 g	4.04 g	8.95 g
Scutellaria baicaensis roots	60 g	9.18 g	7.18 g
Scutellaria lateriflora (whole plant)	60 g	6.54 g	4.08 g

Example 2. Inhibition of COX-2 and COX-1 Peroxidase Activity by Plant Extracts from various *Scutellaria* species

The bioassay directed screening process for the identification of specific COX-2 inhibitors was designed to assay the peroxidase activity of the enzyme as described below.

Peroxidase Assay. The assay to detect inhibitors of COX-2 was modified for a high throughput platform (Raz). Briefly, recombinant ovine COX-2 (Cayman) in peroxidase buffer (100 mM, TBS, 5 mM EDTA, 1 μM Heme, 0.01 mg epinephrine, 0.094% phenol) was incubated with extract (1:500 dilution) for 15 minutes. Quantablu (Pierce) substrate was added and allowed to develop for 45 minutes at 25°C. Luminescence was then read using a Wallac Victor 2 plate reader. The results are set forth in Table 2.

Table 2 sets forth the inhibition of enzyme by the organic and aqueous extracts obtained from five plant species, including the roots of two *Scutellaria* species and extracts from three other plant species, which are comprised of structurally similar Free-B-Ring Flavonoids. Data is presented as the percent of peroxidase activity relative to the recombinant ovine COX-2 enzyme and substrate alone. The percent inhibition by the organic extract ranged from 30% to 90%.

Table 2. Inhibition of COX-2 Peroxidase activity by *Scutellaria* species

Plant Source	Inhibition of COX-2 by organic extract	Inhibition of COX-2 by aqueous extract
Scutellaria orthocalyx (root)	55%	77%
Scutellaria baicaensis (root)	75%	0%
Desmodium sambuense (whole plant)	55%	39%
Eucaluptus globulus (leaf)	30%	10%
Murica nana (leaf)	90%	0%

Comparison of the relative inhibition of the COX-1 and COX-2 isoforms requires the generation of IC_{50} values for each of these enzymes. The IC_{50} is defined as the concentration at which 50% inhibition of enzyme activity in relation to the control is achieved by a particular inhibitor. In the instant case, IC_{50} values were found to range from 6 to 50 μg/mL and 7 to 80 μg/mL for the COX-2 and COX-1 enzymes, respectively, as set forth in Table 3. Comparison, of the IC_{50} values of COX-2 and COX-1 demonstrates the specificity of the organic extracts from various plants for each of these enzymes. The organic extract of *Scutellaria lateriflora* for example, shows preferential inhibition of COX-2 over COX-1 with IC_{50} values of 30 and 80 μg/mL, respectively. While some extracts demonstrate preferential inhibition of COX-2, others do not. Examination of the HTP fractions and purified compounds from these fractions is necessary to determine the true specificity of inhibition for these extracts and compounds.

Table 3. IC_{50} Values for Human and Ovine COX-2 and COX-1

Plant Source	IC_{50} Human COX-2 (μg/mL)	IC_{50} Ovine COX-2 (μg/mL)	IC_{50} Ovine COX-1 (μg/mL)
Scutellaria orthocalyx (root)	ND	10	10
Scutellaria baicaensis (root)	30	20	20
Scutellaria lateriflora (whole plant)	20	30	80
Eucaluptus globulus (leaf)	ND	50	50
Murica nana (leaf)	5	6	7

Example 3. HTP Fractionation of Active Extracts

Organic extract (400 mg) from *Scutellaria baicaensis* roots was loaded onto a prepacked flash column. (2 cm ID x 8.2 cm, 10g silica gel). The column was eluted using a Hitachi high throughput purification (HTP) system with a gradient mobile phase of (A) 50:50 EtOAc:hexane and (B) methanol from 100% A to 100% B in 30 minutes at a flow rate of 5 mL/min. The separation was monitored using a broadband wavelength UV detector and the fractions were collected in a 96-deep-well plate at 1.9 mL/well using a Gilson fraction

collector. The sample plate was dried under low vacuum and centrifugation. DMSO (1.5 mL) was used to dissolve the samples in each cell and a portion (100 μL was taken for the COX inhibition assay.

Aqueous extract (750 mg) from *Scutellaria baicaensis* roots was dissolved in water (5 mL), filtered through a 1 μm syringe filter and transferred to a 4 mL HPLC vial. The solution was then injected by an autosampler onto a prepacked reverse phase column (C-18, 15 μm particle size, 2.5 cm ID x 10 cm with precolumn insert). The column was eluted using a Hitachi high throughput purification (HTP) system with a gradient mobile phase of (A) water and (B) methanol from 100% A to 100% B in 20 minutes, followed by 100% methanol for 5 minutes at a flow rate of 10 mL/min. The separation was monitored using a broadband wavelength UV detector and the fractions were collected in a 96-deep-well plate at 1.9 mL/well using a Gilson fraction collector. The sample plate was freeze-dried. Ultra pure water (1.5 mL) was used to dissolve samples in each cell and a portion of 100 μL was taken for the COX inhibition assay.

Example 4. Inhibition of COX Peroxidase Activity by HTP Fractions from various *Scutellaria* Species

Individual bioactive organic extracts were further characterized by examining each of the HTP fractions for the ability to inhibit the peroxidase activity of both COX-1 and COX-2 recombinant enzymes. The results are depicted in Figure 1, which depicts the inhibition of COX-2 and COX-1 activity by HTP fractions from *Scutellaria baicaensis* isolated as described in Example 1 and 3. The profile depicted in Figure 1 shows a peak of inhibition that is very selective for COX-2. Other Scutellaria sp. including *Scutellaria orthocalyx* and *Scutellaria lateriflora* demonstrate a similar peak of inhibition (data not shown). However, both the COX-1 and COX-2 enzymes demonstrate multiple peaks of inhibition suggesting that there is more than one molecule contributing to the initial inhibition profiles.

Example 5. Isolation and Purification of the Active Free-B-Ring Flavonoids from the Organic Extract of *Scutellaria orthocalyx*

The organic extract (5 g) from the roots of *Scutellaria orthocalyx*, isolated as described in Example 1, was loaded onto prepacked flash column (120 g silica, 40 μm particle size 32-60 μm, 25 cm x 4 cm) and eluted with a gradient mobile phase of (A) 50:50 EtOAc:hexane and (B) methanol from 100% A to 100% B in 60 minutes at a flow rate of 15 mL/min. The fractions were collected in test tubes at 10 mL/fraction. The solvent was evaporated under vacuum and the sample in each fraction was dissolved in 1 mL of DMSO and an aliquot of 20 μL was transferred to a 96 well shallow dish plate and tested for COX inhibitory activity. Based on the COX assay results, active fractions #31 to #39 were combined and evaporated. Analysis by HPLC/PDA and LC/MS showed a major compound with a retention times of 8.9 minutes and a MS peak at 272 m/e. The product was further purified on a C18 semi-preparation column (25 cm x 1 cm), with a gradient mobile phase of (A) water and (B) methanol, over a period of 45 minutes at a flow rate of 5 mL/minute. Eighty eight fractions were collected to yield 5.6 mg of light yellow solid. Purity was determined by HPLC/PDA and LC/MS, and comparison with standards and NMR data. ^{1}H NMR: δ ppm. (DMSO-d6) 8.088 (2H, m, H-3',5'), 7.577 (3H, m, H-2',4',6'), 6.932 (1H, s, H-8), 6.613 (1H, s, H-3). MS: [M+1]+ = 271m/e. The compound has been identified as Baicalein. The IC_{50} of Baicalein against the COX-2 enzyme is 10 μg/mL.

Example 6. COX Inhibition of Purified Free-B-Ring Flavonoids

Several other Free-B-Ring Flavonoids have been obtained and tested at a concentration of 20 μg/mL for COX-2 inhibition activities using the methods described above. The results are summarized in Table 4.

Table 4. Inhibition of COX Enzymatic Activity by Purified Free-B-Ring Flavonoids

Free-B-Ring Flavonoids	Inhibition of COX-1	Inhibition of COX-2
Baicalein	107%	109%
5,6-Dihydroxy-7-methoxyflavone	75%	59%
7,8-Dihydroxyflavone	74%	63%
Baicalin	95%	97%
Wogonin	16%	12%

Example 7. HPLC Quantification of Free-B-Ring Flavonoids in Active Extracts from *Scutellaria orthocalyx* roots, *Scutellaria baicaensis* roots and *Oroxylum indicum* seeds

The presence and quantity of Free-B-Ring Flavonoids in five active extracts from three different plant species have been confirmed and are set forth in the Table 5. The Free-B-Ring Flavonoids were quantitatively analyzed by HPLC using a Luna C-18 column (250 x 4.5 mm, 5 μm) using 0.1% phosphoric acid and acetonitrile gradient from 80% to 20% in 22 minutes. The Free-B-Ring Flavonoids were detected using a UV detector at 254 nm and identified based on retention time by comparison with Free-B-Ring Flavonoid standards. The HPLC chromatograms are depicted in Figure 2.

Table 5. Free-B-Ring Flavonoid Content in Active Plant Extracts

Active Extracts	Weight of Extract	% Extractible from BioMass	Total amount of Flavonoids	% Flavonoids in Extract
Scutellaria orthocalyx (AE)*	8.95 g	14.9%	0.2 mg	0.6%
Scutellaria orthocalyx (OE)*	3.43 g	5.7%	1.95 mg	6.4%
Scutellaria baicaensis (AE)*	7.18 g	12.0%	0.03 mg	0.07%
Scutellaria baicaensis (OE)*	9.18 g	15.3%	20.3 mg	35.5%
Oroxylum indicum (OE)*	6.58 g	11.0%	0.4 mg	2.2%

*AE: Aqueous Extract
*OE: Organic Extract

Example 8. *In vitro* Study of COX Inhibitory Activity of Free-B-Ring Flavonoids from various *Scutellaria* Species

In vitro efficacy and COX-2 specificity of Free-B-Ring Flavonoids isolated from various *Scutellaria* species were tested in cell based systems for their ability to inhibit the generation of arachidonic acid metabolites. Cell lines HOSC that constitutively express COX-2 and THP-1 that express COX-1 were tested for their ability to generate prostaglandin E2 (PGE2) in the presence of arachidonic acid.

COX-2 Cell Based Assay. HOSC (ATCC#8304-CRL) cells were cultured to 80-90% confluence. The cells were trysinized, washed and resuspended in 10 mL at 1 x 10^6 cells/mL in tissue culture media (MEM). The cell suspension (200 μL) was plated out in 96 well tissue culture plates and incubated for 2 hours at 37°C and 5% CO_2. The media was then replaced with new HOSC media containing 1 ng/mL IL-1b and incubated overnight. The media was removed again and replaced with 190 mL HOSC media. Test compounds were then added in 10 μL of HOSC media and were incubated for 15 minutes at 37°C. Arachidonic acid in HOSC media (20 mL, 100 μM) was added and the mixture was incubated for 10 minutes on a shaker at room temperature. Supernatant (20 μL) was transferred to new plates containing 190 μL/well of 100 μM indomethacin in ELISA buffer. The supernatants were analyzed as described below by ELISA.

COX-1 Cell Based Assay. THP-1 cells were suspended to a volume of 30 mL (5x10^5 cells/mL). TPA was added to a final concentration of 10 nM TPA and cultured for 48 hours to differentiate cells to macrophage (adherent). The cells were resuspended in HBSS (25 mL) and added to 96 well plates in 200 mL volume at 5 x 10^5 cells/well. The test compounds in RPMI 1640 (10 μL) were then added and incubated for 15 minutes at 37°C. Arachidonic acid in RPMI (20 μL) was then added and the mixture was incubated for 10 minutes on a shaker at room temperature. Supernatant (20 μL) was added to Elisa buffer (190 μL) containing indomethacin (100 μM). The supernatants were then analyzed by ELISA, as described below.

COX-2 Whole Blood assay. Peripheral blood from normal, healthy donors was collected by venipuncture. Whole blood (500 μL) was incubated with test compounds and extracts for 15 minutes at 37°C. Lipopolysaccharide (from *E. coli* serotype 0111:B4) was

added to a final concentration of 100 μg/mL and cultured overnight at 37°C. Blood was centrifuged (12,000 x g) and the plasma was collected. Plasma (100 μL) was added to methanol (400 μL) to precipitate proteins. Supernatants were measured for PGE2 production by ELISA. This procedure is a modification of the methods described by Brideau *et al.* (1996) Inflamm. Res. 45:68-74.

COX-1 Whole Blood Assay. Fresh blood was collected in tubes not containing anti-coagulants and immediately aliquoted into 500 μL aliquots in siliconized microcentrifuge tubes. Test samples were added, vortexed and allowed to clot for 1 hour at 37°C. Samples were then centrifuged (12,000 x g) and the plasma was collected. The plasma (100 μL) was added to methanol (400 μL) to precipitate proteins. Supernatants were measured for TXB2 production by ELISA. This procedure is a modification of the methods described by Brideau *et al.* (1996) Inflamm. Res. 45:68-74.

ELISA Assays. Immunolon-4 ELISA plates were coated with capture antibody 0.5-4 μg/mL in carbonate buffer (pH 9.2) overnight at 4°C. The plates were washed and incubated for 2 hours with blocking buffer (PBS + 1% BSA) at room temperature. The plates were washed again and test sample (100 μL) was added and incubated for 1 hour at room temperature while shaking. Peroxidase conjugated secondary antibody was added in a 50 μL volume containing 0.5-4 mg/mL and incubated for 1 hour at room temperature while shaking. The plates were then washed three times and TMB substrate (100 μL) was added. The plates were allowed to develop for 30 minutes, after which the reaction was stopped by the addition of 1 M phosphoric acid (100 μL). The plates were then read at 450 nm using a Wallac Victor 2 plate reader.

Cytotoxicity. Cellular cytotoxicity was assessed using a colorimetric kit (Oxford biochemical research) that measures the release of lactate dehydrogenase in damaged cells. Assays were completed according to manufacturers' directions. No cytotoxicity has been observed for any of the tested compounds.

The results of the assays are set forth in Table 6. The data is presented as IC_{50} values for direct comparison. With reference to Table 6, IC_{50} values are generally lower for COX-1 than COX-2. Whole blood was also measured for the differential inhibition of PGE2

generation (a measure of COX-2 in this system) or thromboxane B2 (TXB2) (a measure of COX-1 activation). Referring to Table 6, these studies clearly demonstrate specificity for COX-2 inhibition from the organic extracts in two of the three species tested. Possible reasons for this discrepancy are the fundamental differences between immortalized cell lines that constitutively express each of the enzymes and primary cells derived from whole blood that that are induced to express COX enzymes. Primary cells are the more relevant model to study the *in vivo* inflammation process.

Table 6. Inhibition of COX Activity in Whole Cell Systems

	Cell Line Based Assay		Whole Blood Assay	
Plant Source	**IC_{50} COX-2**	**IC_{50} COX-1**	**IC_{50} COX-2**	**IC_{50} COX-1**
Scutellaria orthocalyx (root)	50 μg/mL	18 μg/mL	10 μg/mL	>50 μg/mL
Scutellaria baicaensis (root)	82 μg/mL	40 μg/mL	20 μg/mL	8 μg/mL
Scutellaria lateriflora (whole plant)	60 μg/mL	30 μg/mL	8 μg/mL	20 μg/mL

Example 9. *In vivo* Study of COX Inhibitory Activity of Free-B-Ring Flavonoids from various *Scutellaria* Species

In vivo inhibition of inflammation was measured using two model systems. The first system (ear swelling model) measures inflammation induced directly by arachidonic acid. This is an excellent measure of COX-2 inhibition, but does not measure any of the cellular events which would occur upstream of arachidonic acid liberation from cell membrane phospholipids by phospholipase A2 (PLA2). Therefore, to determine how inhibitors function in a more biologically relevant response the air pouch model was employed. This model utilizes a strong activator of complement to induce an inflammatory response that is characterized by a strong cellular infiltrate and inflammatory mediator production including cytokines as well as arachidonic acid metabolites.

Ear Swelling Model. The ear swelling model is a direct measure of the inhibition of arachidonic acid metabolism. Arachidonic acid in acetone is applied topically to the ears of mice. The metabolism of arachidonic acid results in the production of proinflammatory

mediators produced by the action of enzymes such as COX-2. Inhibition of the swelling is a direct measure of the inhibition of the enzymes involved in this pathway. The results are set forth in Figure 3, which shows the effects of three extracts delivered either orally by gavage or interperitoneally (IP) at two time points (24 hours and 1 hour). Free-B-Ring Flavonoids isolated from *S. baicaensis* inhibited swelling when delivered by both IP and gavage although more efficacious by IP. (Figures 3A and B). Free-B-Ring Flavonoids isolated from S. orthocalyx inhibited the generation of these metabolites when given IP, but not orally, whereas extracts isolated from *S. lateriflora,* while being efficacious *in vitro*, had no effect *in vivo* (data not shown).

Air Pouch Model. Because Free-B-Ring Flavonoids isolated from *S. baicaensis* were the more efficacious in the ear swelling model, they were also examined using the air pouch model of inflammation. Briefly, an air pouch was created on the back of the mice by injecting 3 mL of sterile air. The air pouch was maintained by additional injections of 1 mL of sterile air every other day for a period of one week. Animals were dosed using the same methods and concentrations described for the ear-swelling model and injected with Zymosan (into the air pouch) to initiate the inflammatory response. After four hours, the fluid within the pouch was collected and measured for the infiltration of inflammatory cells, myeloperoxidase (MPO) activity (a measure of cellular activation, degranulation), and tumor necrosis factor-α (TNF-α) production (a measure of activation). The results are set forth in Figure 4.

Figure 4A shows the total number of cells collected from the air pouch fluid. While there was a strong response that was inhibited by controls (indomethacin), Free-B-Ring Flavonoids isolated from *S. baicaensis* did not inhibit the infiltration of the inflammatory cells (chemotaxsis). Even though the chemotactic response was not diminished, the fluid was examined to determine whether the infiltrating cells have become activated by measuring MPO activity and TNF-α production. Figure 4B and 4C demonstrate that both MPO activity and TNF-α production are significantly reduced when the extract is administered IP, but not by gavages. These data suggest that although the Free-B-Ring Flavonoids do not inhibit the chemotactic response induced by complement activation they are still effective at reducing

inflammation through the prevention of release and production of pro-inflammatory mediators.

Arachidonic Acid induced ear swelling. The ability of Free-B-Ring Flavonoids to directly inhibit inflammation *in vivo* was measured as previously described (Greenspan *et al.* (1999) J. Med. Chem. 42:164-172; Young *et al.* (1984) J. Invest. Dermat. 82:367-371). Briefly, groups of 5 Balb/C mice were given three dosages of test compounds as set forth in Figure 4 either interperitoneally (I.P.) or orally by gavage, 24 hours and 1 hour prior to the application of arachidonic acid (AA). AA in acetone (2 mg/15 μL) was applied to the left ear, and acetone (15 μL) as a negative control was applied to the right ear. After 1 hour the animals were sacrificed by CO_2 inhalation and the thickness of the ears was measured using an engineer's micrometer. Controls included animals given AA, but not treated with anti-inflammatory agents, and animals treated with AA and indomethacin (I.P.) at 5 mg/kg.

Air pouch model of inflammation. Air pouch models were adapted from the methods of Rioja *et al.* (2000) Eur. J. Pharm. 397:207-217. Air pouches were established in groups of 5 Balb/C mice by injection of sterile air (3 mL) and maintained by additional injections of 1 mL every 2 days for a period of six days. Animals were given three dosages of test compounds as shown in Figure 4 either I.P. or orally by gavage, 24 hours and 1 hour prior to the injection of 1% Zymosan (1 mL) into the pouch. After 4 hours, the animals were sacrificed by CO_2 inhalation and the air pouches were lavaged with sterile saline (3 mL). The lavage fluid was centrifuged and the total number of infiltrating cells determined. Supernatants were also retained and analyzed for myleoperoxidase (MPO) activity and the presence of TNF-α by ELISA as measures of activation.

Example 10. Development a Standardized Free-B-Ring Flavonoid Extract from *Scutellaria* Species

Scutellaria orthocalyx (500 mg of ground root) was extracted twice with 25 mL of the following solvent systems. (1) 100% water, (2) 80:20 water:methanol, (3) 60:40 water:methanol, (4) 40:60 water:methanol, (5) 20:80 water:methanol, (6) 100% methanol, (7) 80:20 methanol:THF, (8) 60:40 methanol:THF. The extract solution was combined,

concentrated and dried under low vacuum. Identification of chemical components was carried out by High Pressure Liquid Chromatography using a PhotoDiode Array detector (HPLC/PDA) and a 250 mm x 4.6 mm C18 column. The chemical components were quantified based on retention time and PDA data using Baicalein, Baicalin, Scutellarein, and Wogonin standards. The results are set forth in Table 7.

Table 7. Quantification of Free-B-Ring Flavonoids Extracted from *Scutellaria orthocalyx* Using Different Solvent Systems

Extraction Solvent	Weight of Extract	% Extractible from BioMass	Total amount of Flavonoids	% Flavonoids in Extract
100% water	96 mg	19.2%	0.02 mg	0.20%
water:methanol (80:20)	138.3 mg	27.7%	0.38 mg	0.38%
water:methanol (60:40)	169.5 mg	33.9%	0.78 mg	8.39%
water:methanol (40:60)	142.2 mg	28.4%	1.14 mg	11.26%
water:methanol (20:80)	104.5 mg	20.9%	0.94 mg	7.99%
100% methanol	57.5 mg	11.5%	0.99 mg	10.42%
methanol:THF (80:20)	59.6 mg	11.9%	0.89 mg	8.76%
methanol:THF (60:40)	58.8 mg	11.8%	1.10 mg	10.71%

Scutellaria baicaensis (1000 mg of ground root) was extracted twice using 50 mL of a mixture of methanol and water as follows: (1) 100% water, (2) 70:30 water:methanol, (3) 50:50 water:methanol, (4) 30:70 water:methanol, (5) 100% methanol. The extract solution was combined, concentrated and dried under low vacuum. Identification of the chemical components was carried out by HPLC using a PhotoDiode Array detector (HPLC/PDA), and a 250 mm x 4.6 mm C18 column. The chemical components were quantified based on retention time and PDA data using Baicalein, Baicalin, Scutellarein, and Wogonin standards. The results are set forth in Table 8.

Table 8. Quantification of Free-B-Ring Flavonoids Extracted from *Scutellaria baicaensis* Using Different Solvent Systems

Extraction Solvent	Weight of Extract	% Extractible from BioMass	Total amount of Flavonoids	% Flavonoids in Extract
100% water	277.5 mg	27.8%	0.01 mg	0.09%
water:methanol (70:30)	338.6 mg	33.9%	1.19 mg	11.48%
water:methanol (50:50)	304.3 mg	30.4%	1.99 mg	18.93%
water:methanol (30:70)	293.9 mg	29.4%	2.29 mg	19.61%
100% methanol	204.2 mg	20.4%	2.73 mg	24.51%